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I/We request the grant of a patent on the basis of this application

Signature Wendy Anne Filler

10 March, 1999

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0181-966 5721

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Expression

The present invention relates *inter alia* to the provision of increased polypeptide
5 expression.

The human Hsp70A gene has been sequenced by Hunt C. and Morimoto R.I.(1985) *Proc.Natl.Acad.Sci. USA* **82**, 6455-6459.) This gene encodes an mRNA containing a 5'-untranslated region (5'UTR) of 215 bases. As for most of the vertebrate Hsp
10 mRNAs, the base composition of the human Hsp70 5'UTR is rich in guanosine and cytosine (~62%) (Joshi C.P. and Nguyen H.T. (1995) *Nucleic Acids Res.* **23**, 541-549) suggesting that the human Hsp70 5'UTR has a high potential to form secondary structures in this region. It is believed that the function of the human Hsp70 5'UTR has never been previously studied.

15

In contrast, the *Drosophila* Hsp70 5'UTR has been extensively studied (Di Nocera P.P. and Dawid I. (1983) *Proc.Natl.Acad.Sci.USA* **80**, 7095-7098; Bonner J.J. *et al.* (1984) *Cell* **37**, 979-991; McGarry T.J. and Lindquist S. (1985) *Cell* **42**, 903-911; Hultmark D. *et al.* (1986) *Cell* **44**, 429-438; Lindquist S. and Petersen R. (1990)
20 *Enzyme* **44**, 147-166). The sequence of the *Drosophila* Hsp70 5'UTR (Ingolia T.D. *et al* (1980) *Cell* **21**, 669-679) has no significant homology with the human Hsp70 5'UTR. The lack of secondary structure in the *Drosophila* 5'UTR region due to a rich adenosine composition (~50%) allows efficient translation of this mRNA during heat shock (Hess M.A and Duncan R.F. (1996) *Nucleic Acids Res.***12**, 2441-2449).

25

Experimental studies with both *in vivo* and *in vitro* systems clearly demonstrate that mRNA with a high potential to form stable secondary structures in the 5'UTR tends to be inefficiently translated (Kozac M.(1991) *J. Biol. Chem.* **266**, 19867-19870; Kozac M. (1991) *J. Cell Biol.* **115**, 887-903). Moreover, structural motifs in the
30 5'UTR can provide sites for the binding of proteins which can act as negative

regulators of translation (Gray N.K. and Hentze M.W. (1994) *EMBO J.* **13**, 3882-3891; Stripecke R. *et al* (1994) *Mol.Cell.Biol.* **14**, 5898-5909).

WO94/11521 is directed to providing inducible expression by using a bovine hsp70 promoter. The promoter may be associated with a human or bovine hsp70 5' untranslated region.

Surprisingly the present inventors have now identified a molecule with a high potential to form secondary structures that can provide increased efficiency of translation.

According to the present invention there is provided a DNA molecule that can be transcribed to provide an RNA molecule having an untranslated region that can provide an increased efficiency of translation of a polypeptide (relative to that obtainable when said untranslated region is absent) when operably linked to a coding region encoding said polypeptide; wherein said DNA molecule does not encode a mammalian hsp70.

Preferably the increased efficiency of translation is an increase of at least 10%. More preferably it is an increase of at least 100%. Most preferably it is an increase of at least 500%.

The use of the present invention to provide significantly increased efficiency of translation (and thereby to provide increased expression) contrasts with the invention disclosed in WO94/11521, for example. This discloses the possibility of using a human hsp70 5' untranslated region, but not for obtaining an increased efficiency of translation. In any event, as indicated above, WO94/11521 is specifically directed to the bovine hsp70 promoter and its use in promoting inducible expression. It is preferred that the bovine promoter hsp70 described in WO94/11521 is not used in the present invention. The human hsp70 promoter may for example

be used in the present invention. Promoters which are not hsp promoters may also be used and are often preferred. Further, and in contrast to WO94/11521, heat shock is not required to increase the protein expression in the present invention.

- 5 The untranslated region of the present invention is preferably located upstream of the coding sequence of the RNA molecule – i.e. it is a 5' untranslated region (a 5'UTR).

Preferred DNA molecules of the present invention comprise:

- 10 a) the sequence:

5'-ataacggctagcctgaggagctgctgcgacagtccactaccttttcgagagtgactcccgtgtcccaa
ggcttccagagcgaaacctgtgcggctgcaggcaccggcgcgctcgagttccggcggtccggaaggaccgagctctt
ctcgcggatccagtgttccgttccagcccccaatctcagagccgagccgacagagagcaggggaaccgc-3',

- 15 [On transcription this sequence will produce an mRNA molecule having the following 5'UTR :

5'-

auaacggcuagccugaggagcugcugcgacaguccactaccuuuuucgagagugacucccguuguccaa
ggcuucccagagcgaaaccugugcggcugcaggcaccggcgcgucgaguuuccggcguccggaaggaccg
20 agcucuucucgcggauccaguguuuccgagcccccaaucucagagccgagccgacagagagcagg
gaaccgc-3']

- b) the complement of the sequence given in a); or

- 25 c) a sequence having substantial sequence identity with a sequence as defined in a) or b) above.

Thus a DNA molecule having a specific sequence that can be transcribed to provide the untranslated region of the present invention is within the scope of the present

- 30 invention (see a) above).

The complement of this sequence is also within the scope of the present invention (see b) above) since the DNA molecule will normally be double-stranded. In any event the complement is useful in designing probes or primers or in providing
5 antisense molecules (which can be used to reduce expression if expression levels become too high). Furthermore, cDNA (which is also within the scope of the present invention) will comprise the complement.

DNA molecules having substantial sequence identity with molecules described in a)
10 and b) above may be used in a similar manner to said molecules and are therefore also within the scope of the present invention (see c) above).

The UTR of the present invention is preferably capable of providing heat-shock responsiveness to the expression of a coding sequence in a given expression
15 system. However this is not essential since the untranslated region can provide increased expression even in the absence of a heat shock response.

Desirably the untranslated region of the present invention has a G+C content of greater than 50%. More desirably this is greater than 55% or greater than 60%.
20 High G+C contents are often associated with an increased tendency to form stable secondary structures.

Preferred DNA molecules of the present invention are those that can be transcribed to provide an RNA molecule having an untranslated region that can provide an
25 increased efficiency of translation of a polypeptide (relative to that obtainable when said untranslated region is absent) when operably linked to a coding region encoding said polypeptide; wherein said DNA molecule does not encode human hsp70 and wherein said untranslated region has a ΔG of below -10 kCal/mol.

For the purposes of the present invention ΔG can be calculated using the RNA structural prediction program MFOLD (Zuker M. and Jacobson A.B. (1995) Nucleic Ac. Res. (23) 2791-2798). Predicted ΔG values may be calculated using the program located at the internet: <http://mfold1.wustl.edu/~mfold/mRNA/form1.cgi>

5

Preferably ΔG is below -30 kCal/mol or below -40 kCal/mol. More preferably ΔG is below -50 kCal/mol. Generally speaking, the lower the ΔG value, the greater the degree of secondary structure likely for a given polynucleotide region.

- 10 Increased translation efficiency can be achieved with the present invention in a wide variety of different systems. Indeed the present inventors have provided a 5'UTR upstream of the coding sequence of two very different reporters (firefly luciferase and chloramphenicol-acetyltransferase) and have demonstrated significantly increased expression of the reporter (5- to 10-fold) in normal transfected cell culture
- 15 conditions.

This effect has been obtained in two different promoter contexts (HSP- and SV40-promoter) and in various human cell lines (HepG2, Hep3B, HEK293, WI-38). The 5'UTR did not not modify the level of mRNA but increased the efficiency of

20 translation. This pure translational effect and the fact that the heat shock response is a highly conserved mechanism provide evidence in support of the broad applicability of the present invention.

In principle the expression of any given polypeptide can be increased using the

25 present invention. However it is preferred to use the present inventions to increase the expression of polypeptides that are not heat shock proteins. Most preferably the present invention is used in providing increased expression of polypeptides of relatively high commercial or scientific value. It can for example be used to increase the expression of therapeutic polypeptides. These include interferons, hormones

30 (e.g. insulin), interleukins, erythropoietin, tpa, growth factors, etc. The present

invention can of course also be used to increase the expression of other polypeptides – e.g. polypeptides useful in the agro-alimentary or cosmetic industries

A further aspect of the present invention is the provision of new vectors. These may
5 be derived by modifying known vectors to include a DNA sequence which, on transcription, provides an untranslated sequence of the present invention. This can be done by recombinant DNA technology or by mutagenesis techniques. Alternatively vectors may be constructed *de novo*.

10 Vectors can be used for many purposes – e.g. for amplifying, maintaining or manipulating sequences of interest, for the production of desired gene products, for medicinal purposes etc. Vectors (and nucleic acids) of the present invention may be purified and provided in isolated form if desired. They may be provided in a form substantially free of contaminating proteins.

15 Many different types of vector can be provided, including plasmids, phasmids, cosmids, YACs, PACs and viruses. Viral vectors include bacteriophage vectors. These can be used to generate high titre combinatorial libraries. Using 'phage display many different polypeptides can be expressed (e.g. antibodies/parts thereof). These
20 techniques are described for example by M J Geisow in *Tibtech* 10, 75-76 (1992) and by D. Chiswell *et al* in *Tibtech* 10, 8-84 (1992). Other vectors can be used in addition to those described above.

Whatever vectors are used, it is preferred that they include one or more selectable
25 markers - e.g. drug resistance markers and/or markers enabling growth on a particular medium. In some cases a vector will include a marker that is inactivated when a nucleic acid molecule according to the present invention is inserted into the vector. Here there is desirably at least one further marker, which is different from the marker that is inactivated.

Preferred vectors of the present invention may be introduced into a cell that can then be used to express a desired polypeptide (although cell-free expression systems can also be used). For example, polypeptides can be produced by micro-organisms such as bacteria or yeast, by cultured insect cells (which may be baculovirus-infected), by
5 mammalian cells (such as CHO cells) or by transgenic animals that, for instance, secrete the proteins in milk (see e.g. international patent application WO88/00239). Where glycosylation is desired, eukaryotic expression systems are preferred.

Particularly suitable expression systems are cell lines that can divide in culture and that
10 can be maintained in culture over a long period. These are often referred to as immortal cell lines. Preferred cell lines are mammalian or human cell lines.

Various transcriptional and translational control sequences may be used in expression systems of the present invention. These can be operably linked to a coding sequence
15 encoding a polypeptide to be expressed. The control sequences may be heterologous to the coding sequence. Promoter, operator and/or enhancer sequences may, for example, be provided, as may polyadenylation sites, splice sites, stop and start codons, etc. Polypeptides may initially be expressed to include signal sequences. Different signal sequences may be provided for different expression systems.
20 Alternatively, signal sequences may be absent.

Techniques for manipulating nucleic acids, for expressing and purifying polypeptides, etc. are well known to a person skilled in the art of biotechnology. Such techniques are disclosed in standard text-books, such as in Sambrook *et al* [*Molecular Cloning* 2nd
25 Edition, Cold Spring Harbor Laboratory Press (1989)]; in Old & Primrose [*Principles of Gene Manipulation* 5th Edition, Blackwell Scientific Publications (1994)]; and in Stryer [*Biochemistry* 4th Edition, W H Freeman and Company (1995)].

The present invention is useful in medicine (both in human treatment and in
30 veterinary treatment). It can be used to treat an existing condition or can be used for

prophylactic treatment. In particular, the present invention is useful for treating a disorder involving a deficiency in the expression of a polypeptide. It will therefore be appreciated that the present invention can be used in gene therapy, especially for treating disorders arising due to mutations affecting the expression of a single
5 polypeptide (although it is generally applicable and can also be used to treat disorders affecting the expression of a plurality of polypeptides). Gene therapy may be used, for example, in the treatment of cancer, cardiovascular disorders, cystic fibrosis, etc.

- 10 Treatment of a disorder involving a deficiency in the expression of a polypeptide can be performed by providing a patient with a DNA molecule of the present invention that encodes said polypeptide, with a vector comprising said DNA molecule, or with a cell comprising said DNA molecule or vector. Expression of the polypeptide within the patient can then be used to compensate for, or at least to reduce the deficiency.
- 15 The DNA molecule or the vector can be allowed to integrate into a patient's genome.

Suitable techniques for introducing a nucleic acid molecule or vector into a patient include topical application of the 'naked' nucleic acid in an appropriate vehicle. The nucleic acid may be present together with a pharmaceutically acceptable excipient,
20 such as phosphate buffered saline (PBS). One technique involves particle bombardment (which is also known as 'gene gun' technology and is described in US Patent No. 5371015). Here inert particles (such as gold beads coated with a nucleic acid) are accelerated at speeds sufficient to enable them to penetrate a surface of a recipient (e.g. skin) by means of discharge under high pressure from a
25 projecting device. (Particles coated with a nucleic acid molecule of the present invention are within the scope of the present invention, as are devices loaded with such particles.) Other methods of administering the nucleic acid directly to a recipient include ultrasound, electrical stimulation, electroporation and microseeding. Particularly preferred is the microseeding mode of delivery. This is described in US-
30 5,697,901.

- Nucleic acid molecules of the present invention may also be administered by means of specialised delivery vectors useful in gene therapy. Gene therapy approaches are discussed for example by Verme *et al*, Nature 1997, 389:239-242. Both viral and
- 5 non-viral systems can be used. Viral based systems include retroviral, lentiviral, adenoviral, adeno-associated viral, herpes viral and vaccinia-viral based systems. Non-viral based systems include direct administration of nucleic acids and liposome-based systems.
- 10 A nucleic acid sequence of the present invention may even be administered by means of transformed cells. Such cells include cells harvested from a subject. The nucleic acid molecules of the present invention can be introduced into such cells *in vitro* and the transformed cells can later be returned to the subject. The nucleic acid molecules need not be introduced into the cells as vectors, since non-vector nucleic
- 15 acid molecules can be introduced. Some such molecules may integrate into nucleic acid already present in a cell by homologous recombination events. A transformed cell may, if desired, be grown up *in vitro* and one or more of the resultant cells may be used in the present invention. Cells can be provided at an appropriate site in a patient by known surgical or microsurgical techniques (e.g. grafting, micro-injection,
- 20 etc.)

Another way of treating a deficiency in the expression of a polypeptide comprises providing a patient with a DNA molecule that can be transcribed to provide the untranslated region of the present invention. This molecule can be provided in a

25 manner to allow it to become operably linked with a sequence already present in the patient that encodes said polypeptide.

A further way of treating a deficiency in the expression of a polypeptide comprises providing a patient with an RNA molecule coding for said polypeptide, which RNA

molecule is prducable by transcribing a DNA molecule of the present invention. The RNA molecule can then be translated *in vivo* to provide the polypeptide.

A still further way of treating a deficiency in the expression of a polypeptide, 5 comprises providing a patient with the polypeptide, wherein the polypeptide has been produced using an expression system of the present invention.

The present invention is also useful in providing DNA vaccines. The direct injection of gene expression cassettes into a living host transforms a number of cells into 10 factories for the production of the introduced gene products. Expression of these delivered genes has important immunological consequences and may result in the specific immune activation of the host against expressed antigens. Although vaccines produced by recombinant DNA technology are safer than traditional vaccines, which are based on attenuated or inactivated bacteria or viruses, they are 15 often poorly immunogenic. Placing an untranslated region of the present invention upstream of the coding sequence of a gene to be delivered in a DNA vaccine can significantly increase expression and can therefore increase immunogenicity. Due to the highly conserved mechanism of heat shock response an increase in polypeptide expression can be expected in every tissue where the gene is delivered. DNA 20 vaccines can be designed to prevent viral, bacterial and parasitic infections (e.g. diphtheria, malaria, leishmaniasis, toxoplasmosis, schistosomiasis, cryptosporidiosis, tuberculosis, HIV, HSV, influenza virus, hepatitis A, B and C), but can also be used for treating cancer, immune-related diseases or for contraceptive purposes. All of these applications are within the scope of the present invention.

25 When used in medicine, the nucleic acid molecules, vectors, polypeptides and cells discussed above will usually be in the form of a pharmaceutically acceptable composition. One or more pharmaceutically acceptable carriers may be present in such a composition. A pharmaceutical composition within the scope of the present 30 invention may be adapted for administration by any appropriate route, for example

by the oral (including buccal or sublingual), rectal, nasal, topical (including buccal, sublingual or transdermal), vaginal or parenteral (including subcutaneous, intramuscular, intravenous or intradermal) routes. Different drug delivery systems can be used to administer pharmaceutical compositions, depending upon the
5 desired route of administration. Drug delivery systems are described, for example, by Langer (*Science* **249**, 1527–1533 (1991)) and by Illum and Davis (*Current Opinions in Biotechnology* **2**, 254–259 (1991)). In summary, it will be appreciated that the present invention can be used to manufacture medicaments for use in the treatment of one or more of the disorders discussed herein.

10

In addition to the uses discussed above, the invention is of broad applicability for research purposes.

Cell transfection is a technique classically used in research to study the function of a
15 polypeptide. Moreover, many cellular screenings are performed on cells transfected so as to express a given polypeptide. This technique is also used to study the function of a promoter using reporters (e.g. luciferase, chloramphenicol-acetyl-transferase, β -galactosidase etc.) Providing an untranslated region of the present invention upstream of the coding sequence of a reporter gene can significantly
20 increase the expression of a polypeptide of interest. The sensitivity of such experiments can therefore be increased.

Increased polypeptide expression is also useful in many other research applications where large amounts of a given polypeptide need to be synthesized. For example it is useful in structural studies (crystallography, NMR, etc), for the production of
25 antibodies or fragments thereof (which can be used for example in purification or in binding studies), or for high throughput screening.

The present invention is also useful for diagnostic purposes. For example, it can be used to increase the provision of antibodies or fragments thereof useful in
30 diagnosing the presence of a moiety associated with a particular disorder.

The present invention will now be described by way of example only with reference to the accompanying drawings, wherein:

Figure 1 shows the effect of the human Hsp70 5'UTR on the expression of a luciferase reporter driven by the human HSP70 promoter. The human HSP70 promoter was cloned upstream of the coding sequence of the firefly luciferase gene in the absence or presence of the 5' UTR (plasmids A and B respectively). For both plasmids the 3'UTR was the HSP70 3'UTR. HepG2 cells were transfected with these two chimeric constructs and the levels of luciferase were compared either under normal conditions or after a 30 min heat-shock at 42°C.

Figure 2 shows the effect of the human Hsp70 5'UTR on the expression of luciferase in various cell lines. The same constructs (A and B) were transfected in three other human cell lines (Hep3B, HEK293, WI-38) and the luciferase levels were compared under normal cell culture conditions.

Figure 3 shows the effect of the human Hsp70 5'UTR on the translational efficiency of the chloramphenicol-acetyl-transferase mRNA. The SV40 promoter was cloned upstream of the coding sequence of the chloramphenicol-acetyl-transferase (CAT) gene in the absence or presence of the human Hsp70 5' UTR (plasmids H and I respectively). HepG2 cells were transfected with these two constructs and the levels of CAT mRNA and activity were measured.

Figure 4 shows the effect of the human Hsp70 5'UTR on the expression of luciferase in the presence of the SV40 3'UTR. The Hsp70 promoter was cloned upstream of the coding sequence of the luciferase in the absence or presence of the human Hsp70 5'UTR (plasmids C and D respectively), for both plasmids the 3'UTR was the SV40 3'UTR. The constructs (C and D) were transfected in three human cell lines (HepG2, HEK293, Hep3B) and the luciferase levels were compared under normal cell culture conditions.

Figure 5 shows a comparison of the effect of the human Hsp70 5'UTR with the human Grp78 5'UTR on the expression of the luciferase. The SV40 promoter was cloned upstream of the coding sequence of the luciferase in the presence of the human Hsp70 5'UTR (plasmid F) or of the human Grp78 5'UTR (plasmid G) or in the absence of any 5'UTR (plasmid E). HepG2 cells were transfected with these three constructs and the luciferase levels were compared under normal cell culture conditions.

Figure 6 is provided for reference purposes and gives the hsp70 5'UTR sequences from various different species.

Figure 7 is also provided for reference purposes. It shows the sequence identity of the human hsp70A 5'UTR with the hsp70 5'UTR from various other species and with the human HSP70B 5'UTR.

Examples

Materials

Cell culture medium (BME and MEM), penicillin, streptomycin, trypsin-EDTA solution, versene, non-essential amino acids and restriction enzymes were obtained from Gibco, Life Technologies, Inc. Foetal bovine serum (heat inactivated) was from HyClone, Culture flasks (TPP T150) and 60mm culture dishes (Falcon) were purchased from Becton Dickson. Plasmids were obtained from Promega. The HepG2, Hep3B, HEK293 and WI-38 cell lines were obtained from the American Type Culture Collection.

Methods

Plasmids constructs

HSP70 reporter vectors were generated using pGL3 promoter vector plasmid for the firefly luciferase assay, or pCAT3 promoter vector plasmid for the chloramphenicol

acetyl transferase (CAT) assay were purchased from PROMEGA. Human HSP70 promoter ,human HSP70 5'UTR and 3'UTR were PCR-amplified (Advantage GC genomic PCR kit, Clontech) from a human genomic bank (Clontech). Oligonucleotides were designed from the human hsp70A gene (Hunt C. and Morimoto R.I.(1985) Proc.Natl.Acad.Sci.USA 82, 6455-6459) (GenBank : g184416).

Sequence of the human Hsp70 5'UTR and regions (bold characters) utilized to design oligonucleotides for the PCR reaction :

10 5'-

ataacggctagcctgaggagctgctgcgacagtccactaccttttcgagagtgactcccgtgtccaaggctcc
cagagcgaacctgtgcggctgcaggcaccggcgcgctcgagttccggcgctccggaaggaccgagctcttctcgcg
atccagtgtccgtttccagcccccaatctcagagccgagccga **agagagcaggggaaccgc**-3'

15 For the the human Grp78 5'UTR primers were designed using the sequence published by Ting J. and Lee A.S. (1988) DNA (4) 275-278 (GenBank : g183644).

The 5'UTRs were inserted between the Hind III and Nco I sites, HSP70 promoter between Bgl II and Nco I sites. The human Hsp70 3'UTR was inserted between the
20 XbaI and BamH1 sites.

All the constructs were sequence-checked.

Cell culture conditions

25 HepG2 and WI-38 cell lines were maintained in BME supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin-glutamine. For HepG2 cells, medium was supplemented with 1% non essential amino acids, 1% sodium. Hep3B and HEK293 were maintained in MEM (Gibco Life Technologies, Inc) supplemented with 10% fetal bovine serum (HyClone) and 1% penicillin-streptomycin-glutamine. Cells were
30 maintained at 37°C in humidified air containing 5% CO₂.

Transient transfections

HepG2, Hep3B, 293 and WI-38 cells were transiently transfected with the indicated construct and the internal control pRL-TK vector for luciferase assay or pSve- β Gal
5 vector for CAT assay, using the calcium phosphate co-precipitation method.

Quantification of reporter genes activities

Reporter gene activities were quantified 48 hours after transfection. In heat-shock experiments, 48h hours after transfection the cells were heat-shocked at 42°C for 40
10 minutes and then maintained 4 hours at 37°C before luciferase activities were measured.

Luciferase activities were quantified using the Dual Luciferase Assay (Promega). Values were normalised with the renilla luciferase activity expressed from pRL-TK.
15 CAT activities were normalised with the β galactosidase activity expressed from pSve- β Gal .

CAT mRNA quantification

The ^{33}P CAT probe was synthesized with linearized pTRI-CAT vector (CAT Direct™:
20 CAT mRNA detection kit, Ambion) using MaxiScript™ : *in vitro* transcription kit (Ambion), according to the manufacturer's protocol.

Confluent transfected cells were washed with phosphate-buffered saline (PBS). Cells were lysed in TRIZOL (Gibco, Life Technologies, Inc). Total mRNA was extracted using a 24:1 v/v of chloroform/isoamyl alcohol. Total mRNA was pelleted
25 with an equal volume of isopropanol. Pellet was washed with a cold 70% ethanol solution and then solubilized in lysis buffer of Direct Protect™ kit (Lysate Ribonuclease Protection Assay, Ambion).

CAT mRNAs were quantified by a lysate ribonuclease protection assay using Direct
30 Protect™ kit (Ambion) according to the manufacturer's protocol.

Protected fragments were resolved in 5% poly-acrylamide gels containing 8M urea and radioactivity was quantified using a Phosphorimager (STORM, Molecular Dynamics).

5

Example 1: Effect of the 5'UTR of the human HSP70 mRNA on the expression of a luciferase reporter driven by the HSP70 promoter (Figure 1).

The human HSP70 promoter was cloned upstream of the coding sequence of the firefly luciferase gene in the absence or presence of the 5' UTR (plasmids A and B
10 respectively). HepG2 cells were transfected with these two chimeric constructs and the levels of luciferase were compared either in normal conditions or after a 30 min heat-shock at 42°C. The presence of the 5'UTR of the human HSP70 mRNA by itself strongly increased the level of expression of the luciferase. Under both conditions (normal and heat-shock) a similar 9-fold stimulation was observed in the presence of
15 the 5'UTR demonstrating that this increase in the luciferase expression is inherent to this 5'UTR and independent of the stress. Therefore, it can be expected that the human Hsp70 5'UTR can be used to enhance the expression of a wide variety of genes in normal cellular conditions.

20 Example 2: Effect of the 5'UTR of the human HSP70 mRNA on the expression of luciferase in various cell lines (Figure 2).

HSPs are ubiquitous proteins and the heat-shock response is a highly conserved mechanism. Therefore, a similar effect of the human Hsp70 5'UTR was expected in other cell lines. Three other human cell lines were transfected with the same
25 constructs. Hep3B is a hepatic cell line very close to HepG2. The two other cell lines tested, WI-38 and HEK293, are from different origins. WI-38 is a fibroblast-like cell line derived from embryonic lung tissue and HEK293 is a transformed primary embryonal kidney cell line. As shown in Figure 2, the 5'UTR of the human HSP70 mRNA increased the expression of the luciferase gene in the three cell lines tested.
30 An ~9-fold stimulation was obtained in the presence of the 5'UTR in Hep3B cells

comparable to the stimulation observed in HepG2. In WI-38 and HEK293 cells the effect of the 5'UTR was less (a ~ 5-fold stimulation) but still significant, showing that this 5'UTR effect is not cell type specific. Therefore, it can be expected that this sequence can be used in a broad spectrum of applications where genes are
5 expressed in various cell.

Example 3: Effect of the human HSP70 5'UTR on the translational efficiency of the chloramphenicol- acetyl-transferase mRNA (Figure 3).

A higher level of luciferase expression in the presence of the HSP70 5'UTR can be
10 explained either by a higher level of luciferase mRNA (due to an increase either in transcription or in mRNA stability) or by a more efficient translation of the luciferase mRNA. In order to distinguish between these two possible mechanisms, the mRNA levels in the absence or in the presence of the HSP70 5'UTR (plasmid H and I, respectively) were measured in transfected HepG2 cells. In this experiment CAT
15 gene was used as reporter and was driven by the SV40. As previously observed with the luciferase gene, the presence of the HSP70 5'UTR cloned upstream of the coding sequence of the CAT gene increased (~10-fold) the CAT activity. Moreover, this higher level of CAT was achieved without any significant change in the CAT mRNA level in the presence of the HSP70 5'UTR. This result shows that the HSP70
20 5'UTR increases the translational efficiency of the mRNA independently of the reporter gene or the promoter used. Therefore, it can be expected that this human Hsp70 5'UTR property of increasing translational efficiency can be obtained for a wide variety of genes and promoters and can thus be used for a broad range of applications.

25

Example 4: Effect of human Hsp70 5'UTR on the expression of the luciferase in presence of the SV40 3'UTR (Figure 4).

The 3'UTR present downstream of the reporter coding sequence in commercially available plasmids is frequently the 3'UTR of the SV40 large T antigen. This viral
30 3'UTR is known to allow a high level of expression of the reporter in transfection

experiments. In order to determine if the human HSP70 5'UTR can increase the expression of the luciferase with this heterologous SV40 3'UTR we generated two vectors containing the SV40 3'UTR in the presence or absence of the human HSP70 5'UTR (plasmid C and D respectively). As expected (Figure 4) the SV40 large T antigen 3'UTR allowed a high level of expression of the luciferase gene in the three cell lines tested. A 5- to 10-fold higher level of luciferase was observed in presence of this viral 3'UTR in comparison to the levels of expression obtained with the human HSP70 3'UTR (Figures 1 and 2). Nevertheless, the presence of the human HSP70 5'UTR was still able to increase the level of the luciferase expression by a 2-fold factor in the three cell lines tested.

Example 5: The effect on translation is not a common property of all stress protein 5'UTR (Figure 5).

In order to compare the effect of the HSP70 5'UTR with another stress protein 5'UTR, the human GRP78 5'UTR was used. The human GRP78 5'UTR has a similar length as the HSP70 5'UTR (221bp versus 215pb for HSP70), and both of them are equally G + C rich (63%). Using the MFOLD program (Zuker M. and Jacobson A.B. (1995) Nucléic Ac. Res. (23) 2791-2798) to calculate the stability of these two 5'UTRs, a similar high ΔG value was found (~ -60 kCal/mol), suggesting that these two 5'UTRs form structures of comparably high stability. Therefore the GRP78 5'UTR was an interesting 5'UTR to compare to the HSP70 5'UTR. As shown in Figure 5, this 5'UTR (plasmid G) does not modify the level of expression of the luciferase gene. This result shows that the effect obtained with the HSP70 5'UTR is not a common property of all stress protein 5'UTRs.

D finitions

For the avoidance of doubt, certain terms used herein are further defined below. Similar terms should be construed accordingly.

"Polypeptid "

This means any moiety having a plurality of amino-acids joined together by peptide bonds. It includes proteins and peptides.

"About"

- 5 When used in connection with a numerical value this term allows for a margin either side of the value. Preferably the margin is +/- 10 % of the figure. more preferably it is +/- 5 %

"Sequence identity"

- 10 For the purposes of the present invention, sequence identity may be determined, for example, by using the ALIGN program (version 2.0). This calculates a global alignment of two sequences. (See Myers and Miller,(1989) CABIOS, 4, 11-17). Gap penalties: -16/-4. For information see <http://www.infobiogen.fr/services/menuserv.html>

15

"Substantial Sequence identity"

This term is used to include polynucleotide sequences having at least 50% sequence identity with a given polynucleotide sequence. Preferably the degree of sequence identity is at least 75%. Sequence identities of at least 90%, at least 95% or at least

- 20 99% are most preferred.

"Heat Shock"

This is an increase in temperature which is sufficient to induce the heat shock response. Classically for cells a shift from 37°C to 42°C for 30 minutes is used to

- 25 induce a heat shock.

"Increased Efficiency of Translation"

This means that a greater degree of translation to provide active polypeptides is obtained from a given number of mRNA molecules than would otherwise be the case.

30

Remarks

The foregoing description of the invention is merely illustrative thereof and it should therefore be appreciated that various variations and modifications can be made without departing from the spirit or scope of the invention as set forth in the
5 accompanying claims.

Where preferred or optional features are described in connection with particular aspects of the present invention, they shall be deemed to apply *mutatis mutandis* to other aspects of the invention unless the context indicates otherwise.

10

All documents cited herein are hereby incorporated by reference, as are any citations referred to in said documents.

Claims

1. A DNA molecule that can be transcribed to provide an RNA molecule having an untranslated region that provides an increased efficiency of translation of a polypeptide when operably linked to a region encoding said polypeptide; wherein said DNA molecule does not encode a mammalian Hsp70.
2. A DNA molecule according to claim 1 that does not comprise the bovine hsp70 promoter.
3. A DNA molecule according to any preceding claim; wherein said untranslated region is at least 175 nucleotides long.
4. A DNA molecule according to any preceding claim; wherein said untranslated region is at least 200 nucleotides long.
5. A DNA molecule according to any preceding claim; wherein said untranslated region is about 215 nucleotides long.
6. A DNA molecule according to any preceding claim; wherein said untranslated region is a 5' untranslated region.
7. A DNA molecule according to any preceding claim, comprising:
 - a) the sequence:
5'ataacggctagcctgaggagctgctgcgacagtccactaccttttcgagagtgactcccgtgtcccaaggcttccc
agagcgaacctgtgcggctgcaggcaccggcgcgctcgagttccggcgctccggaaggaccgagctcttctcgcg
atccagtgttcggttccagcccccaatctcagagccgagccgacagagagcaggaaccgc-3',
 - b) the complement of the sequence given in a), or

c) a sequence having substantial sequence identity with a sequence as defined in a) or b) above

5 8. A DNA molecule according to any preceding claim wherein said untranslated region has a ΔG of below -10 kCal/mol.

9. A DNA molecule according to any preceding claim; wherein said sequence has a ΔG that is below -30 kCal/Mol

10

10. A DNA molecule according to any preceding claim; wherein said sequence has a ΔG that is below -40 kCal/Mol

11. A DNA molecule according to any preceding claim; wherein said untranslated
15 region has a ΔG of below -50 kCal/Mol

12. A DNA molecule according to any preceding claim; wherein expression of said polypeptide is heat shock responsive.

20 13. An RNA molecule obtainable by transcribing a DNA molecule according to any of claims 1 to 12.

14. A vector comprising a DNA molecule according to any of claims 1 to 12.

25 15. An expression system comprising a DNA molecule according to any of claims 1 to 12 or a vector according to claim 14.

16. An expression system according to claim 15 which comprises one or more cells.

17. An expression system according to claim 16 comprising one or more eukaryotic cells.
18. An expression system according to claim 16 comprising one or more
5 mammalian cells.
19. An expression system according to claim 16 comprising one or more human cells
- 10 20. An expression system according to claim 15 which is a cell free expression system
21. A method of obtaining a polypeptide comprising expressing the polypeptide using an expression system according to any of claims 15 to 20 and, optionally,
15 purifying the polypeptide.
22. A method according to claim 21 comprising the step of providing the expression system with a heat shock.
- 20 23. A polypeptide when obtained via a method according to claim 21 or claim 22.
24. A method of treating a deficiency in the expression of a polypeptide, comprising providing a patient with a DNA molecule as described in any of claims 1 to 12 which encodes said polypeptide, with a vector comprising said DNA molecule,
25 or with a cell comprising said DNA molecule or vector.
25. A method of treating a deficiency in the expression of a polypeptide, comprising providing a patient with a DNA molecule that can be transcribed to provide the untranslated region defined in any of claims 1 to 12; wherein said

24

molecule is provided in a manner to allow it to become operably linked with a sequence already present in the patient which encodes said polypeptide.

26. A method of treating a disorder (e.g. an infection) treatable by providing an increased immune response, comprising providing a patient with a vaccine comprising a DNA molecule as described in any of claims 1 to 12 or comprising a vector including said DNA molecule.

27. A method according to claim 24 or 25; wherein a DNA molecule or vector is provided under conditions allowing it to integrate within the patient's genome.

28. A method according to claim 24; wherein a cell is provided under conditions allowing it to be maintained within the patient.

29. A method according to claim 28 wherein said cell is a cell that has been removed from the patient and has been modified prior to being reintroduced to the patient.

30. A method of treating a deficiency in the expression of a polypeptide, comprising providing the patient with an RNA molecule according to claim 13 or with a polypeptide according to claim 23.

31. A pharmaceutically acceptable composition comprising a DNA molecule according to any of claims 1 to 12, an RNA molecule according to claim 13, a polypeptide according to claim 23 or a cell as described in any of claims 16 to 19.

32. A vaccine comprising a DNA molecule according to any of claims 1 to 10, or a vector including said DNA molecule.

33. The use of a DNA molecule according to any of claims 1 to 12, of an RNA molecule according to claim 13, of a vector according to claim 14, or of an expression system according to any of claims 15 to 20, in achieving increased expression of a polypeptide.

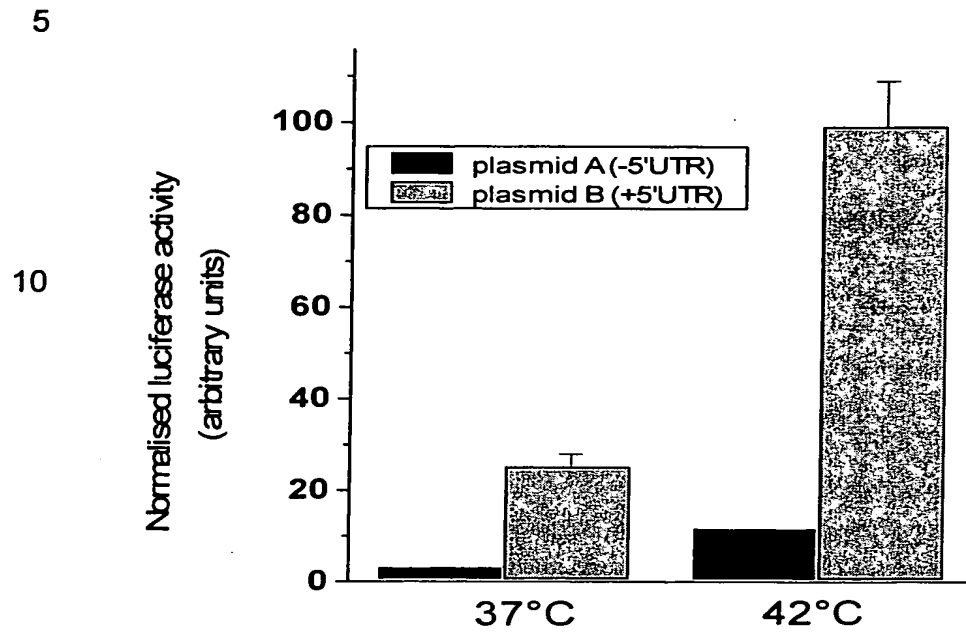
5

34. The invention as substantially herein before described with reference to the accompanying drawings and examples.

Abstract**Expression**

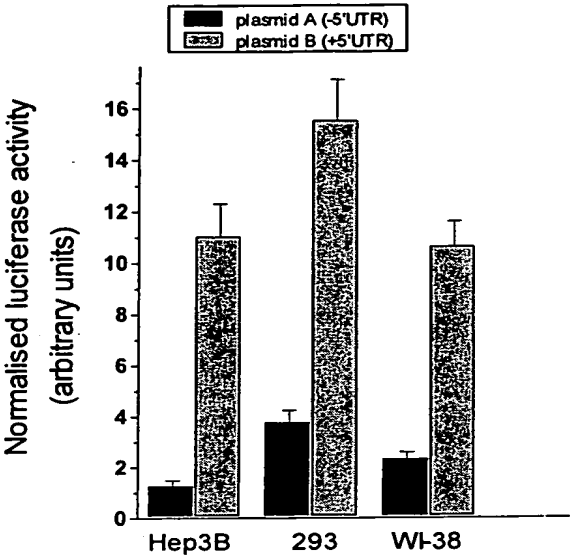
5 Untranslated regions associated with the heat shock response can be used to obtain increased efficiency of translation of polypeptides that are not necessarily normally associated with the heat shock response. This allows the development of greatly improved expression systems. The invention is also useful, for example, in the treatment of a patient suffering from a deficiency in the expression of a polypeptide
10 and in the provision of vaccines.

Figur 1

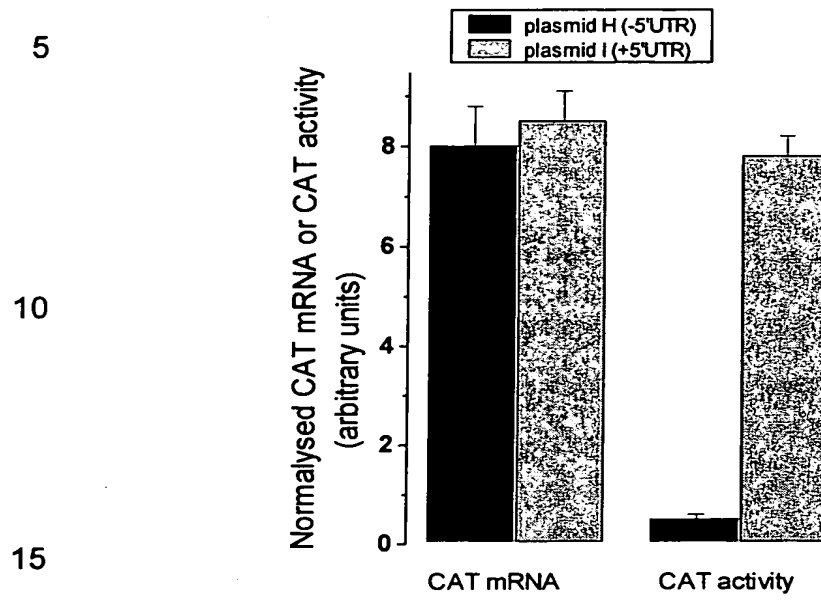


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Figure 2



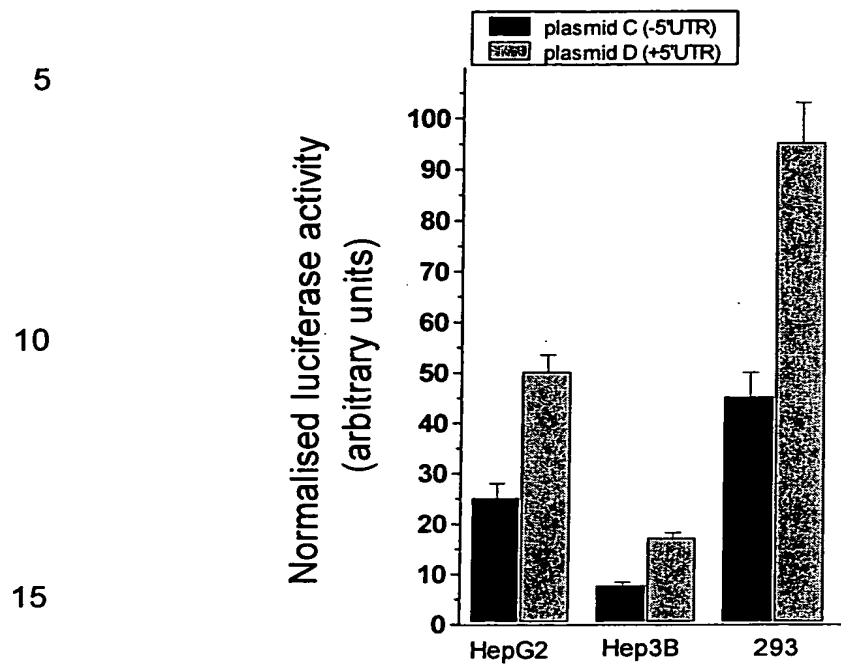
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Figur 3

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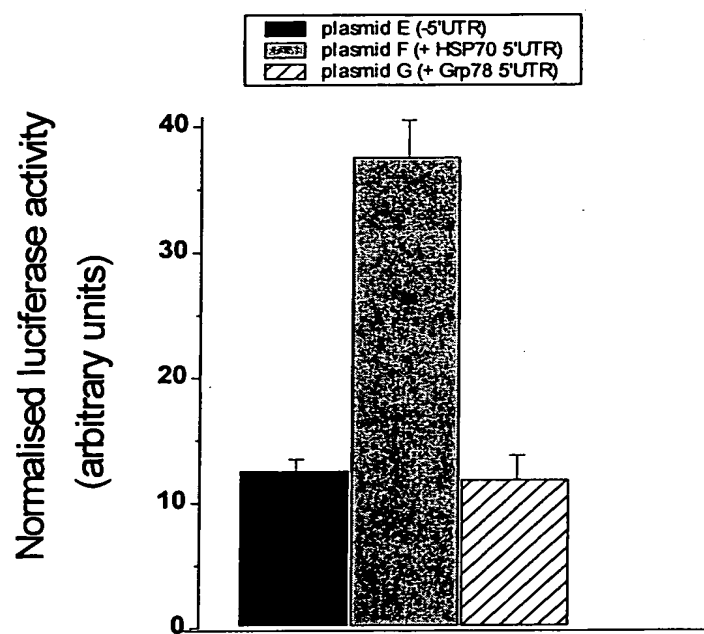
30

Figure 4



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Figure 5



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Figur 6**HSP70 5'UTR SEQUENCES**

5

Human HSP70AHunt C. and Morimoto R.I. (1985) *Proc.Natl.Acad.Sci. USA* **82**, 6455-6459

ataacggctagcctgaggagctgctgcgacagtccactaccttttcgagagtgactcccgtgtccaaggctccca
gagcgaacctgtgcggtgcaggcacccggcgctcgagttccggcgctccggaaggaccgagctcttcgcggat
10 ccagtgtccgtttccagccccaatctcagagccgagccgacagagagcaggggaaccgc

Human HSP70BSchiller et al (1988) *J.Miol.Biol.* **203**,97-105

15 agcagatccggccgggctggcggcagagaaaccgcagggagagcctcactgctgagcgcccctcgacgcgggc
ggcagcagcctccgtggcctccagcatccgacaagaagcttcagcc

20 Rat HSP70

Mestrl,R., Chi,S.H., Sayen,M.R. and Dillmann,W.H.

Biochem. J. 298 Pt 3, 561-569 (1994)

ctcctcctaattctgacagaaccagtttctggttcactcgagagaagcagagaagcagagcaagcggcgcggtcc
25 gaacctcgggcaagaccagcctctccagagcatccccacgcgaagcgacccttctccagagcataccccagc
ggagcgcacccttccccagagcatccccgcccgaagcgcaaccttccagaagcagaccgcagcgac

Chicken HSP70

Morimoto,R.I., Hunt,C., Huang,S.-Y., Berg,K.L. and Banerji,S.S.

30 J. Biol. Chem. 261, 12692-12699 (1986)

cggcagatcgcgccgcagacagcagcgagaagcgggaggagacgtgactgagcgagcaagtgactg
gcggagcgagtggctgactgaccaagaggaatctatcatc

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Mouse HSP70

Hunt,C. and Calderwood,S.B.

5 Gene 87, 199-204 (1990)

aagctactcagaatcaaactctggttccatccagagacaagcgaagacaagagaagcagagcgagcggcgcggtc
ccgatcctcggccaggaccagccttccccagagcatccacgccgaggagcgcaaccttcccaggagcatccctgc
cgcgaggagcgcaacttccccggagcatccacgccgaggagcgagccttccagaagcagagcgcgggcg

10

African Green monkey HSP70

Sainis,I., Angelidis,C., Pagoulatos,G. and Lazaridis,I.

FEBS Lett. 355 (3), 282-286 (1994)

15 Gaattccggttctagagcgtggctcccgttgtccgaggcttcccagagcgaacctgtgcggctgcaggcaccagcg
ccgttgagtttccggcggtccggaggactgagctctgtcacgggtcccgtccggttccagtcccgaatctcggagc
ggacgagacagcaggggcaccggc

Bos taurus Angus HSP70

20 ACCESSION U02892

NID g414974

AUTHORS Grosz,M.D. and Skow,L.C.(unpublished)

gccgcctgaggagaaacagcagcctggagagagctgataaaacttacggcttagtccgtgagagcagttccgcag
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This sequence is only available in Genbank data base (g414974)

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